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Leukocyte CD18 receptors may be a better target than ICAM-1 ligands for reducing histologic evidence of cellular and vascular rejection in the rabbit

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Abstract Building evidence suggests that blocking the ICAM-1/CD18 interaction may affect the course of graft rejection. Treatment with monoclonal antibody (mAb) to CD18 was compared to antibody to ICAM-1 in a rabbit heterotopic heart transplant model to determine whether blocking the leukocyte receptor for ICAM-1, CD18, was more effective than antibody targeting of the ligand for ICAM-1. Following transplantation, 28 recipient rabbits were randomized to receive either placebo, mAb to CD18, or mAb to ICAM-1 for 7 days until sacrifice. The cellular rejection grade and percentage of arteries with vascular rejection were significantly lower in animals treated with anti-CD18 than with anti-ICAM-1. As assessed by histology, antibody treatment was more effective in reducing both cellular and vascular rejection when directed at the leukocyte receptor CD18 than the ICAM-1 ligand. These findings suggest that other ICAM ligands may play an active role in the immune response and that CD18 may be important for migration of lymphocytes through myocardium.

Key words Rejection, CD18 receptors, rabbit heart · Rejection, ICAM-1 ligands, rabbit heart · CD18 receptors, rejection, rabbit heart · ICAM-1 ligand, rejection, rabbit heart · Rabbit, rejection, heart

Introduction

In primates, use of the monoclonal antibody (mAb) R6.5 to ICAM-1 has been documented to prolong graft survival in both renal and cardiac transplantation without other immunosuppression [4, 10]. This has prompted the initiation of clinical trials of this antibody in transplantation [12]. The histology of cardiac grafts treated with antibody to ICAM-1 has not been fully described, but it has been suggested that lymphocytic infiltration of the graft persists in the face of antibody treatment [10].

Our laboratory has previously demonstrated a significant reduction in the extent and pattern of lymphocytic infiltration into rabbit cardiac grafts by treatment with mAb 60.3 directed against the common β subunit of the CD11/CD18 receptors for ICAM-1 [23]. In the current study, we utilized the same rabbit model to pursue the differences in cellular rejection grade and histology when antibody blockade was directed at the ligand (ICAM-1) versus the receptor (CD18) side of this interaction.

Methods

Animal preparation

A model of cervical heterotopic cardiac transplantation was employed using New Zealand White rabbits as recipients (1.5–3.0 kg) and Stauffland rabbits (1–2 kg) as donors. This donor-recipient combination reliably results in concomitant severe cellular rejection and significant transplant arteritis by post-transplant day 9.

The operative technique has previously been reported [23]. All experiments were performed in accordance with "The Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for Care and Use of Laboratory Animals" (NIH Publication No. 80–23, revised 1978).

Experimental protocol

Recipient rabbits were randomized to one of three treatment groups. Group 1 animals ($n = 11$) received saline injections and served as controls; group 2 animals ($n = 10$) received mAb 60.3 to CD18 at 1 mg/kg per day; group 3 animals ($n = 7$) were treated with mAb RR1/1.1.1 (RR1/1) to ICAM-1 at 1 mg/kg per day. Each antibody was administered intravenously on a daily basis for 7 days post-transplant. No other immunosuppressive drugs were given. In the interests of antibody conservation, the first dose was given 4 h post-transplant, once recipient graft survival had been assured following the transplant procedure. All animals were sacrificed on post-transplant day 7 in order to assess the transplanted heart at a point prior to end-stage rejection with coronary circulation intact.

Monoclonal antibodies

Monoclonal antibody 60.3 (Bristol-Meyers-Squibb, Seattle, Wash.; P. Beatty, Fred Hutchinson Cancer Research Center; and R. Winn, J. Harlan, University of Washington, Seattle, Wash.), a murine IgG_{2a} directed against human CD18, has been previously shown to have cross reactivity in rabbits. The plasma levels and half life of this antibody in rabbits at the dose utilized have been previously documented at this institution [20].

Monoclonal antibody RR1/1.1.1 (R. Rothlein, R. Barton, Boehringer-Ingelheim Pharmaceuticals, Ridgefield, Conn.), a murine IgG_1 that binds to the first and second domains of human ICAM-1 [6, 27], has been previously shown to have crossreactivity in rabbits as demonstrated by in vitro binding to rabbit cells, in vivo functional efficacy in cardiac [3] and pulmonary ischemia-reperfusion phenomena in rabbits [14], and visualization of antibody bound to endothelium and leukocytes in rabbit tissues [22].

The specificity of mAb RR1/1 was confirmed in our laboratory by binding to ICAM-1-transfected CHO cells with absence of binding VCAM-1- or E-selectin-transfected or control CHO cells (CHO cells a gift of R. Lobb, Biogen, Cambridge, Mass.). As a functional assay, a serum sample drawn from an unoperated rabbit 24 h following injection of 1 mg/kg of mAb RR1/1 was found to inhibit homotypic aggregation of PMA-stimulated JY EBV-transformed B lymphoblastoid cells at a 1:4 dilution [22].

Flow cytometry was performed by the invesigators that confirmed strong and equivalent binding of both antibodies to rabbit mononuclear cells. Both antibodies have been purified and shown to be suitable for intravenous injection.

On standard immunocytochemistry using secondary horse anti-mouse antibodies conjugated to avidin-biotin-peroxidase (Vector Laboratories), both mAbs 60.3 and RR1/1 were found to bind to rabbit lymphocytes in paraffin-embedded sections of rabbit lymphoid tissue. Both antibodies were identified on infiltrating mononuclear leukocytes in frozen sections of rejecting donor hearts from untreated control rabbits (Fig. 1).

Histology: rejection

Recipient animals were sacrificed on the 7th day following transplantation. Excised donor and native hearts were fixed in methyl Carnoy's solution (60:30:10 volume mixture of methanol, chloroform, and glacial acetic acid), sectioned transversely into 4-mm-thick sections, and paraffin embedded for hematoxylin-eosin staining. Blinded readings of randomized histology slides were performed twice to ensure the reproducibility of results. Rejection was graded 0–3, depending on the extent of mononuclear cell infiltration, consistent with standardized criteria [1]. Rejection was scored 0, signifying no infiltrates; 1 for mild infiltration of mononuclear cells confined to perivascular spaces; 2 for moderate infiltration with expansion of interstitial spaces by mononuclear cell infiltrates and individual myocyte loss; or 3 for severe, extensive mononuclear cell infiltration with significant myocyte loss. The location of leukocytic infiltrates within perivenular or interstitial sites and whether leukocytes were confined to nodules or present diffusely throughout the myocardium were noted for each transplanted heart in each of the three groups.

Histology: arteritis

For the purposes of this study, the mononuclear cell infiltration of arterial vessels in the transplanted heart was termed arteritis. This designates the inflammatory lesion seen in these hearts and is not meant to imply a necrotic vasculitis. Each of four cross-sections from each heart was treated with Verhoeff's (elastin) stain to differentiate coronary arterioles from venules and capillaries by identifying the internal elastic lamina. The prevalence of arteritis was quantitated by constructing a ratio with the number of affected arteries as the numerator divided by the total number of arterial profiles. Expressing the prevalence of arteritis as a percentage allows correction for encountering the same vessel in serial cross-sections.

A survey of coronary arterial diameters was conducted on hearts from unoperated rabbits whose weight was similar to that of the transplant donors. The normal distribution of coronary arterial and arteriolar diameters was catalogued, using the measurements between the internal elastic laminae (IEL) (defined by a Verhoeff's stain). Two hundred microns was found to be a reproducible point of demarcation between "small" intramyocardial arterioles and "large" epicardial coronary arteries.

In each heart section from the experimental animals, the identified arterial profiles were then separated into "small" and "large" diameter groups depending on whether the IEL-IEL diameter was less than or greater than 200 μm. The prevalence of arteritis was then additionally calculated for small versus large arteries in each heart.

Without vascular perfusion techniques for fixation, it was felt that attempts at quantifying the extent of intimal infiltration within each vessel lumen would not be accurate, so the finding of any intimal infiltration was scored as "positive."

Leukocyte counts

Peripheral blood samples were drawn on the 2nd and 7th days after transplant in all groups to evaluate white blood cell (WBC) counts and differentials.

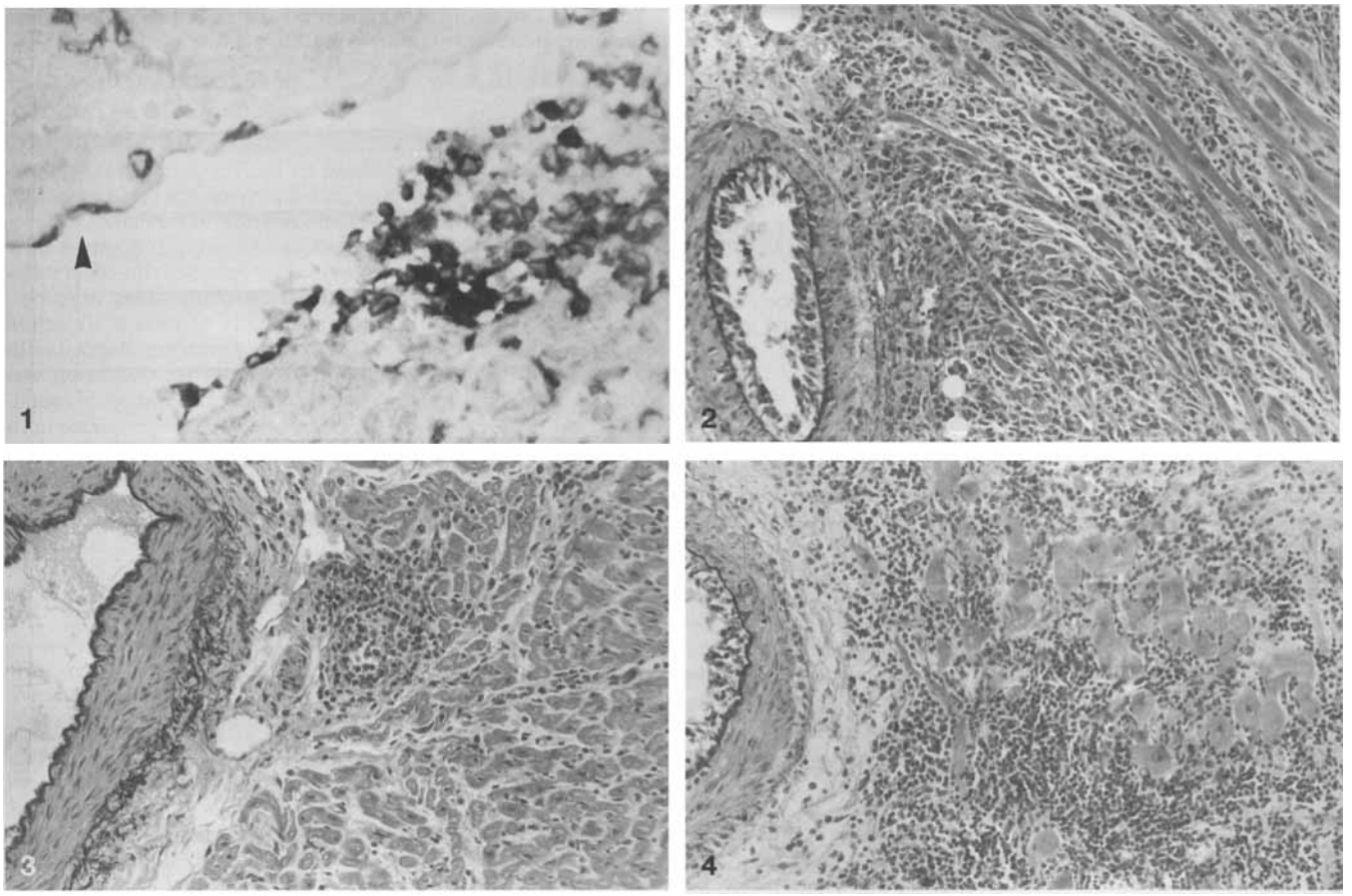

Fig. 1 Frozen section, control group without recipient antibody treatment, donor myocardium, severe rejection. Nuclei are counterstained with methyl green. Monoclonal antibody to human ICAM-1, RR1/1, is visualized by immunocytochemistry (black reaction product) on rabbit venular endothelium (*arrow*) and on invading rabbit mononuclear leukocytes (× 200)

Fig. 2 Control group, donor myocardium, severe rejection, post-transplant day 7. Extensive interstitial infiltrates composed of mononuclear leukocytes are present with extensive myocyte loss. Coronary arteries demonstrate severe transplant arteritis with marked mononuclear infiltration of the intima and scattered cells infiltrating the media (Verhoeff's stain × 200)

Fig. 3 Monoclonal antibody 60.3 (anti-CD18) treatment group, moderate rejection, post-transplant day 7. Infiltrating lymphocytes are tightly collected in perivenular nodules with an obvious reduction in interstitial infiltration. Coronary arteries are free from intimal infiltration (Verhoeff's stain × 200)

Fig. 4 Figure 4: Monoclonal antibody RR1/1 (anti-ICAM-1) treatment group, severe rejection, post-transplant day 7. The pattern of severe, interstitial infiltration of mononuclear cells is indistinguishable from control hearts with severe rejection. Advanced arteritis is evident in a coronary artery (Verhoeff's stain × 200)

Statistical analysis

Rejection scores for cellular and vascular rejection, and WBC counts are reported as a median value and range. Because of the small number of observations, the full range of data from minimum to maximum is presented rather than interquartile ranges.

For ordinal or continuous data, comparisons across groups were done with a Kruskal-Wallis nonparametric one-way analysis of variance. If significant differences were found among the groups, then pairwise comparisons were done using Dunn's procedure. Categorical data were analyzed as two-way tables using Fischer's exact test. Contrasts between paired values for WBC counts on the 2nd and 7th days post-transplant were done using the Wilcoxon nonparametric signed rank test. No multiple comparison correction was made in either the categorical or the paired data testing. Differences were considered statistically significant at a P value less than 0.05.

Results

Cellular rejection

Rejection grade

At sacrifice on postoperative day (POD) 7, the median grade of cellular rejection in control donor hearts was 3.0, signifying severe rejection as expected (Table 1).

Table 1 Histology of cellular and vascular rejection in the three treatment groups

	Treatment groups		
	Control ($N = 11$)	mAb 60.3 (anti-CD18) ($N = 10$)	mAb RR1/1 (anti-ICAM-1) ($N = 7$)
Cellular rejection			
Median rejection grade (range)	3 (1–3)	1.75 (0–2.5)**	3 (2.5–3)**
Rejection grade (number of donor hearts)			
Grade < 3	7*	10*, **	1**
Grade = 3	4	0	6
Pattern of infiltrates (number of donor hearts)			
Nodular	4*	8*, **	0**
Diffuse	7	2	7
Location of intiltrates (number of donor hearts)			
Perivenular	4*	6*, **	0**
Interstitial	7	4	7
Vascular rejection			
Median percentage of arteries with arteritis (range)	4.6 (0–25)	4.9 (0–9)**	20 (4–52)**
Extent of infiltration[a] (number of hearts)			
Intima only	7	10	2
Media and periarterial	4	0	5

* $p < 0.05$ between control and mAb 60.3-treated groups; ** $p < 0.05$ between mAb 60.3-treated and mAb RR1/1-treated groups

[a] Statistical analysis not performed

Donor hearts treated with mAb 60.3 to CD18 had a median cellular rejection score of 1.75, within the range of mild rejection, a 42 % reduction as compared to controls. None of the animals treated with mAb 60.3 evidenced severe rejection on histology, a considerable improvement over control animals and over animals treated with antibody to ICAM-1. The median rejection grade in donor hearts treated with antibody RR1/1 to ICAM-1 was 3.0, severe rejection, significantly higher than hearts treated with antibody to CD18 but similar to untreated controls.

Histology

Untreated control hearts examined on POD 7 evidenced marked infiltration of mononuclear leukocytes into the interstitial spaces with expansion of the interstitial spaces and myocyte loss (Fig. 2).

Donor hearts treated with mAb 60.3 (anti-CD18) had markedly less interstitial infiltration with large areas of myocardium free from infiltration (Fig. 3).

Donor hearts treated with mAb RR1/1 (anti-ICAM-1) demonstrated extensive infiltration of mononuclear cells by POD 7 (Fig. 4), indistinguishable from controls.

The pattern and localization of mononuclear leukocytes within the myocardium were both significantly different between treatment groups. In mAb 60.3-treated hearts, the infiltrates that were present were tightly collected in expanded nodules surrounding venules with the remaining myocardium appearing quite normal (Fig. 3). In contrast, in both control hearts and hearts treated with mAb RR1/1, diffuse interstitial infiltration of invading cells was present throughout the myocardium with some increased numbers of mononuclear cells remaining in the perivenular spaces (Figs. 2, 4).

None of the recipient hearts evidenced any mononuclear cell infiltration.

Vascular rejection

Prevalence

Donor hearts treated with mAb 60.3 showed a significantly lower prevalence of arteritis than hearts treated with mAb RR1/1. The median percentage of arterioles/arteries affected by acute transplant arteritis was similar in the mAb 60.3-treated and control groups, although the range was much narrower in the mAb 60.3-treated group (Table 1).

Histology

The major difference between the groups was that arterial infiltration in animals treated with mAb 60.3 was limited to the intima, whereas in control and mAb RR1/1-treated hearts, individual mononuclear cells were seen invading the media and periarterial spaces as well (Table 1, Fig. 2). Mononuclear cells were found in the media only in the presence of coexisting severe intimal involvement.

In all groups, there was no consistent pattern of arterial involvement that could be differentiated by arterial dimensions. In these sections, "large" epicardial vessels (> 200 μ) were involved with the same frequency as intramyocardial vessels classed into the "smaller" group (data not shown).

Leukocyte counts

On the 2nd day post-transplant, the median WBC count was elevated in the animals treated with antibody to CD18 (15,100; range 13,200–37,300) as compared to both the animals treated with antibody to ICAM-1 (8,500; range 4,100–10,800) and controls (8,900; range 6,500–11,800; $P < 0.05$). Both the absolute neutrophil and lymphocyte counts were also higher on day 2 in the anti-CD18 treatment group than in the other two groups, but this did not reach statistical significance. By POD 7, the median WBC count in the anti-CD18-treated group (13,600; range 7,800–18,500) was still elevated over controls (6,400; range 5,200–8,100; $P < 0.05$), but no longer significantly greater than in the anti-ICAM-1- treated group (9,300; range 2,900–16,900). A persistent rise in the total number of circulating leukocytes following treatment with antibody to CD18 has been previously reported by our laboratory [23].

In control animals, the median WBC count on POD 2 was higher than on POD 7. There was no statistical difference between the WBC counts on day 2 versus day 7 in either of the treatment groups.

Discussion

In this model, treatment with antibody to CD18 was more effective than antibody to ICAM-1 at reducing the histologic evidence of cellular rejection, most likely relating to the effects of these two antibodies on cellular migration through the myocardium. Antibody to CD18 resulted in a marked inhibition of interstitial infiltration by mononuclear leukocytes, whereas the antibody to ICAM-1, RR1/1, exhibited no improvement in histology as compared to controls in this model.

The ICAM-1/LFA-1 interaction is one of several adhesion molecule-receptor interactions responsible for the transendothelial migration and locomotion of lymphocytes. Both antibody to CD18 and this antibody, RR1/1, to ICAM-1 have been found to inhibit the migration of lymphocytes across endothelial monolayers in vitro [17, 29]. However, in our in vivo study, only treatment with antibody to CD18 affected the pattern of mononuclear leukocyte infiltration into the graft. Avidity cycles of LFA-1, more than ICAM-1, are responsible for locomotion on planar bilayers in vitro [7, 8] and this may be more relevant for the in vivo movement of lymphocytes through myocardium than transendothelial migration assays. Inhibition of locomotion, in turn, might explain why treatment with antibody to the CD18 component of LFA-1 appeared to impede progression of lymphocytes into and through the graft interstitium in vivo. Interestingly, a recently presented abstract using a combination of antibodies to CD11a and ICAM-1 in a murine model of cardiac graft tolerance failed to distinguish any diminution in graft infiltration in antibody-treated hearts [2]. The lack of effect of either antibody to CD11a or ICAM-1 on lymphocyte infiltration in that study would support a hypothesis that CD18, and not CD11a or ICAM-1, may be a key determinant for lymphocyte migration through the myocardium.

An essential difference between the two antibodies is that antibody to CD18 inhibits in vitro T cell activation to allogenic vascular endothelium, the hallmark of rejection, more consistently than the same antibody to ICAM-1 used in this study, RR1/1 [24]. Moreover, lymphocyte adherence and activation have been closely linked to phosphorylation of the β subunit of CD18 [13, 28], which can occur, at least initially, without a change in the number of receptor sites [8]. Constitutive expression of CD18 with binding avidity dependent on conformational changes might make CD18 an attractive target for antibody blockade. In contrast, upregulation of ICAM-1 expression may depend on de novo protein synthesis [7], which might, in turn, yield a lower availability of binding sites at the time of administration of antibody if upregulation is not maximal.

ICAM-1 expression has been identified immunocytochemically on intercalated discs in post-transplant human cardiac biopsies during rejection [21]. It may serve as a marker of upregulated target myocytes for invading lymphocytes, or of myocytes in the early stages of apoptosis. Antibody to ICAM-1 might reduce target recognition and subsequent myocytolysis or reduce apoptosis even in the presence of lymphocytic infiltration. This possibility might reconcile our findings of persistent graft infiltration with the previously reported prolongation of graft survival with anti-ICAM-1 treatment [4, 10, 12].

CD18 subtends more than one adhesion ligand. New evidence suggests that ICAM-2, which is constitutively expressed on endothelium without requiring upregula-

tion [18, 19, 26], or ICAM-3, which is better represented than ICAM-1 on resting lymphocytes and monocytes [9, 11], could be as important as ICAM-1 in the initiation of immune responses [5]. Furthermore, ICAM-2 ligands could become facilitated pathways for endothelial transmigration in the presence of ICAM-1 blockade. The potential for antibody to CD18 to block all three known ICAM ligands might explain the better results achieved with anti-CD18 treatment. Recently, a gene mutation of ICAM-1 in donor or recipient mice was found to have no effect in vivo on either mononuclear cell infiltration or cardiac allograft survival [25]. These findings support the premise that other ICAM ligands may be important in the direct interaction of T lymphocytes with vascular endothelium.

Antibody to CD18 has been reported to be more effective than antibody to ICAM-1 (mAb RR1/1) in reducing early neutrophil sequestration and myocardial edema during reperfusion following 3 h of global ischemia in a rabbit cardiac transplant model [3]. Both antibodies were effective in preventing reperfusion-induced contracture, myocardial stunning, and coronary vasoconstriction. To the extent that CD18-mediated activation of neutrophils and release of neutrophil-derived toxic substances may influence the later recruitment of lymphocytes into a transplanted organ, these findings would favor antibodies to CD18 over those to ICAM-1 in our rabbit transplant rejection model.

There may be minor species specific differences between the functions of these two anti-human antibodies in rabbits. In general, the β subunit of CD11/CD18 appears to be better conserved between species than the ICAM-1 molecule [16], suggesting that the antibody to human CD18 might be more effective in this rabbit model. However, both antibodies bind equally to rabbit mononuclear cells on flow cytometry. Also, antibody RR1/1 can be detected on invading lymphocytes and on coronary endothelium in our rejecting rabbit hearts, further confirming its binding to rabbit cells in vivo. It is possible that enough differences might exist between the rabbit ICAM-1 molecule and the mAb RR 1/1 binding site on human ICAM-1 to reduce the full functional efficacy of the antibody even though binding to rabbit constituents appears adequate. However, the fact that this same anti-ICAM-1 antibody has been effective in blocking neutrophil-mediated processes in the rabbit in two other studies [3, 14] would argue that its functional activity has been maintained in these rabbit models.

The differential effects of these two antibodies on vascular rejection also warrant examination. The stable leukocyte counts argue against F_c binding to mAb RR1/1, a murine IgG_1, as a factor in the vascular rejection in this group. The half-life of the $F(ab')_2$ fragment of this antibody is too short to be practical for in vivo use. However, other murine IgG_1 antibodies that bind to coronary vascular endothelium have been used both therapeutically [12] and as isotype controls by our laboratory in this rabbit model without eliciting increased vascular rejection.

Macrophages are felt to play perhaps a more important role in vasculopathic processes than in cellular rejection. Macrophage recruitment through monocyte chemoattractant protein-1, found in nontransplant atherosclerotic plaques in both humans and rabbits [30], may be mediated, in part, by Mac-1 (CD11b/CD18) and p150,95 (CD11c/CD18) and impaired by antibodies to the β_2 integrins [15]. These same factors might be important for macrophage recruitment in transplant vasculitides as well. Antibody to CD18 would block all three of these macrophage receptors and so might result in an effect on macrophage infiltration as well as lymphocyte infiltration, a factor that may be important in vascular rejection.

Among the available antibodies to ICAM-1, antibody RR1/1, as used here, blocks the first and second domains of ICAM-1, affecting only the binding of LFA-1, whereas antibody R6.5 also blocks the Mac-1 binding site in domain 3 [6]. MAb R6.5 has been noted to ameliorate vascular injury in experimental renal rejection [4]. If macrophages are, indeed, key elements in the pathogenesis of this acute transplant arteritis, other anti-ICAM-1 antibodies, such as R6.5, with a broader spectrum for inhibition of monocyte/macrophage adhesion, might be found to be more efficacious in vascular rejection.

In summary, these data, taken together, suggest that the ICAM-CD11/CD18 interaction does play an important role in cardiac graft rejection in this rabbit model and may be a valid target for therapeutic intervention. However, in this model and with these antibodies, blockade of the leukocyte receptor CD18 is more effective than antibody blockade of ICAM-1 in influencing the histologic pattern of rejection in cardiac allografts.

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